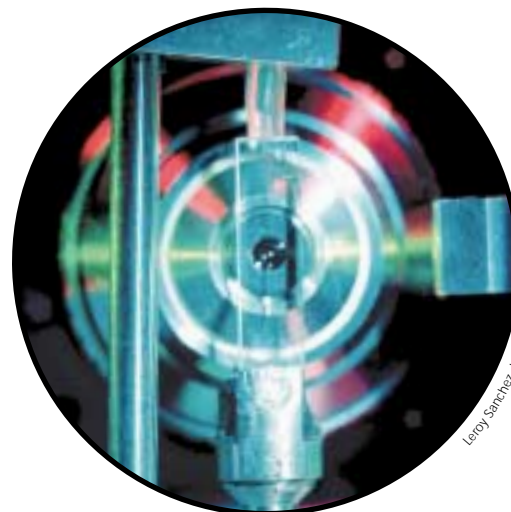


Go with the Flow:



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An Updated Tool for Detecting Molecules

To date, some 4,000 species of bacteria have been identified, 200 of which are pathogenic to humans. Some are a double-edged sword—in the case of *Escherichia coli*, for example, some strains exist as benign and beneficial occupants of the human intestinal system, and others cause potentially life-threatening illnesses. In the event of a bacterial disease outbreak, it's vital for public health officials to know which strain they're dealing with as quickly as possible to be able to track the outbreak's source and limit its extent. (Similar knowledge at the clinical level, usually obtained through a process known as culture and sensitivity, allows for selection of appropriate antibiotics for treatment, as many bacterial strains have developed a resistance to some antibiotics.)

A recent analytical advancement at Los Alamos National Laboratory in New Mexico may have a great influence on rapid bacterial strain identification. A group of Los Alamos scientists led by Richard Keller, Babetta Marrone, and James Jett has built upon earlier flow cytometry technology to create a device that allows public health officials and others to study bacteria at the molecular level, differentiating between individual strains more quickly and with greater accuracy than was possible before.

In the original flow cytometer, developed at Los Alamos in the early 1970s, the

substance being tested is broken down into individual cells, and each cell passes individually in a continuous flow through a laser beam, scattering the light in a characteristic manner. Dyes bound to different parts of the cell emit light, or fluoresce, when passed through the laser.

Sensors within the cytometer measure several parameters, including "low-angle forward scatter intensity," which is approximately proportional to cell diameter, and fluorescence intensities at several wavelengths, which allows for the study of cell components such as total DNA per cell, specific nucleotide sequences, and, by labeling with monoclonal antibodies, specific cellular proteins and other molecules. Flow cytometers are now common in hospitals and public health labs across the country.

A few years ago, the Los Alamos group began refining the capabilities of the flow cytometer so that it could analyze not just a single cell but a single molecule. This development makes the term "cytometer" somewhat of a misnomer, as the new device deals with molecules rather than cells.

Flow Chart

"Single molecule detection is the Holy Grail of analytical chemistry," Jett says. "In addition to instrumental developments, one of the things that helped us . . . was the creation of a whole new family of DNA-bind-

ing dyes that showed a tremendous leap in fluorescence when they bonded with DNA."

The Los Alamos group has used several different nucleic acid stains, including PicoGreen, POPO-3, and TOTO-1, all of which show a 600-fold or larger enhancement in fluorescence when they bind to DNA. But even with the increased fluorescence, relatively little light is emitted when the individual molecules pass through the laser beam. So the group slowed the flow rate from 10 meters per second to 10 millimeters per second, keeping the fragments and the dye bound to them in the light source for a longer period so more photons could be emitted, collected, and measured using a solid-state photon-counting detector.

As stained DNA fragments are run through the flow cytometer, they trigger brief bursts of fluorescence. The size of the burst is directly proportional to the number of dye molecules that bind to the DNA and thus reveals the size of the DNA fragment as measured in base pairs. These bursts are recorded, producing a histogram, a DNA "fingerprint," which is exactly analogous to the electrophoretogram produced by gel electrophoresis. In that technique, DNA-containing samples are purified, then treated with enzymes that cut the DNA at specific points, creating a collection of "clippings" that is characteristic of the organism that produced it. (A later variation of gel

electrophoresis, pulsed-field gel electrophoresis [PFGE], involves alternating an electric current to move particles toward and away from a pair of electrodes to increase size-dependent separation, improving resolution for larger fragments.)

In Los Alamos experiments with purified *E. coli* and *Staphylococcus aureus* samples, DNA histograms were obtained in 10 minutes. Both PFGE and flow cytometry require similar sample preparation times, but even when sample preparation time was factored in, increasing the flow cytometry time to 8 hours, it was still faster than the 24 hours required by PFGE. This is significant even when one considers that flow cytometry runs samples one at a time, something unlikely to happen in a clinical setting, while PFGE can typically run 15 samples on a single gel. The Los Alamos flow cytometer yields results with a much lower uncertainty (2–5% versus 10% for PFGE), is 200,000 times more sensitive than PFGE, and uses picogram quantities of DNA, as opposed to the hundreds of nanograms called for in PFGE. And flow cytometry is far more quantitative than PFGE because it counts individual fragments of DNA, whereas PFGE estimates DNA based on the intensity of the emission from the dye used to visualize the band.

The Los Alamos team has already developed the next model of the flow cytometer using a different DNA-binding dye (SYTOX Orange) and a smaller laser that runs on a five-volt power supply, requires neither water nor forced-air cooling, and emits at approximately the maximum absorption wavelength of the dye—the wavelength to which the dye will be most responsive. A single data-acquisition card in a computer constitutes the data collection system. The laser light is focused on the sample stream delivered by a piece of silicon capillary tubing 30–50 centimeters long and 40 micrometers in interior diameter. A photon-counting silicon avalanche photodiode assembly registers the emitted photons.

Using the Flow

The Los Alamos group has patented the new flow cytometer, and although the patent has yet to be licensed, Jett says the group is in discussion with at least three interested industry groups. Michael Marron, director of biomedical technology at the National Center for Research Resources, says the new flow cytometer shows great promise in a variety of fields where single molecule study would be valuable. “The flow cytometer would be valuable in the study of disease transmission as well as response to drugs,” Marron says. “It would also be valuable in the ongoing study of the human genome, so we’re looking at something useful in both

patient care and treatment, as well as the rapid identification of pathogens.” (Part of the National Institutes of Health in Bethesda, Maryland, the National Center for Research Resources partially funded the group’s research through the National Flow Cytometry Resource, a mechanism at Los Alamos for making state-of-the-art flow cytometric instrumentation available to the biomedical research community.)

Jett adds that the device would also be useful in responding to a bioterrorist attack. In such an event, he says, being able to identify the specific strain of an organism aids in tracing its origin.

Janet Nicholson, associate director for laboratory science at the Centers for Disease Control and Prevention National Center for Infectious Diseases in Atlanta, Georgia, says, “This could be a tremendous tool for quickly identifying the source and type of disease outbreaks, although it’s a technology still in its very early stages, with a lot of work still to be done. Each organism has its own particular fingerprint in PFGE, which [Jett] has shown [to be] true in his flow cytometer as well, so the ability to analyze that fingerprint quickly and with accuracy would be invaluable. You could use it to decide if [an outbreak] was a single-source outbreak or multiple-source; if it was traced to food processing, you could decide if a change in the manufacturing process was needed.”

Nicholson notes that the technology will have to be cost-effective to be valuable. “PFGE requires that you incubate and culture a sample to produce a culture, and many labs don’t do the culture part in order to save money,” she says. “When it comes to health, it shouldn’t be so, but the reality is that any time a lab selects tests, it’s based on the cost in labor, reagents, and time. This new flow cytometer will have to be cost-effective to the point where it really can be found in any lab across the country in order to achieve its full value.”

Jett says the group has found ways to cut the expense of the device from its current estimated cost of \$35,000. “For example,” he says, “we used a microscope objective in the

system that cost \$3,000, but we’ve since found an \$82 aspheric lens that works just as well. We also used a \$6,000 optical mounting system, which would probably be excessive in a commercial system, and we used a motor controller to move optical parts, which you probably wouldn’t use in a commercial system. And our laser cost \$8,000, which commercial production would probably reduce significantly by buying in bulk.”

Other issues to be addressed include how the device will function in a clinical setting and whether it will require purified cultures or whether the cytometer can be programmed to “watch” for specific bacterial indicators within a given sample, searching only for particular biological markers and excluding other tissue elements such as red blood cells or nontarget bacteria, which would be considered contaminants. Says Jett, “[The device] could require a purified culture, but we’re also working on second-color detection, where we can hybridize a probe to measure not only the length of a given fragment, but to know that particular fragments contain particular sequences, and really refine detection.” (Nicholson believes much more work is needed to deal with the complexity of an actual unpurified specimen.) The system will also need access to a large-scale database for rapid comparison of samples with existing patterns. “I don’t know how we’ll handle reference linkups,” Jett admits, referring to this access. “Perhaps through a direct link [to a database], but we could also have a downloadable database, so a hospital dealing with a particular strain could trace it among its patients without relying on the [remote] database. Those are things we’ll address as we proceed.” The operation of the system must also be streamlined and sped up for commercial use, Jett acknowledges.

But none of these are insurmountable obstacles, in Jett’s opinion. Furthermore, he says, given the great value of the system, “I think it makes these goals well worth pursuing.”

Lance Frazer

Suggested Reading

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Kim Y, Jett JH, Larson EJ, Penttila JR, Marrone BL, Keller RA. Bacterial fingerprinting by flow cytometry: bacterial species discrimination. *Cytometry* 36(4):324–332 (1999).

Ambrose WP, Goodwin PM, Jett JH, Van Orden A, Werner JH, Keller RA. Single molecule fluorescence spectroscopy at ambient temperature. *Chem Rev* 99(10):2929–2956 (1999).